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Autoantibodies to neutrophil cytoplasmic antigens (ANCA) do not bind to polymorphonuclear neutrophils in blood

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Autoantibodies to neutrophil cytoplasmic antigens (ANCA) do not bind to polymorphonuclear neutrophils in blood.

Background. Autoantibodies to neutrophil cytoplasmic antigens (ANCA), particularly to proteinase 3 (PR3), are found in the majority of patients with systemic Wegener's granulomatosis. The autoantibodies are widely used as diagnostic markers. Their role in the development and progression of the disease, however, is still under investigation. The primary target of ANCA, PR3, is located in the cytoplasm of polymorphonuclear neutrophils (PMN) or monocytes and is translocated to the cell surface upon stimulation. In patients with Wegener's granulomatosis PR3 is up-regulated most prominently during active disease. Despite the fact that both autoantibodies to PR3 and PMN expressing PR3 are present in patients with Wegener's granulomatosis, there is no evidence for binding of the autoantibodies to PMN. The present study was designed to analyze binding characteristics of autoantibodies to PR3 on PMN.

Methods and Results. PMN of patients with active Wegener's granulomatosis ($N = 10$) were tested for autoantibody binding. Despite high autoantibody titer and PR3 expression on the PMN, no surface-bound IgG was found on PMN ex vivo. When ANCA-containing plasma from patients was incubated with isolated PMN, stimulated to express PR3, again no specific binding of the autoantibody could be detected. Also keeping the samples on ice did not allow surface detection of IgG, ruling out degradation or internalization of the autoantibodies. Only when purified IgG fractions were used, binding to PMN was seen in 14 of 25 patients. Already 1% of plasma, however, was sufficient to greatly reduce the IgG binding. Reduced binding of the IgG fraction was also seen when a larger reaction volume was used.

Conclusion. Our data indicate that autoantibodies to PR3 have a rather low affinity for surface-associated PR3 on PMN. This, in turn, argues against the hypothesis that ANCA contributes to the pathogenesis of the disease by stimulating viable PMN in whole blood.

Key words: Wegener's granulomatosis, ANCA, PMN, proteinase 3, vasculitis.

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According to the original description, Wegener's granulomatosis is a chronic inflammatory disease characterized by necrotizing granulomatous inflammation, particularly of the respiratory tract, by systemic necrotizing vasculitis, and by glomerulonephritis (reviewed in [1]). The etiology and pathogenesis of Wegener's granulomatosis is not yet known. Association with bacterial infections, environmental factors, or certain drugs have been proposed, as have autoimmune phenomena [2–4].

The concurrence of autoantibodies to neutrophil cytoplasmic antigens (ANCA) with Wegener's granulomatosis [5], and the identification of the major target antigen, proteinase 3 (PR3) [6], lead to the hypothesis that ANCA may activate the proinflammatory potential of polymorphonuclear neutrophils (PMN) [7–9]. That PR3 is expressed on PMN of patients with active disease is consistent with this hypothesis [10] as are reports on the presence of activated PMN in the peripheral blood of patients with active Wegener's granulomatosis [11–14].

The potential for tissue destruction and propagation of inflammatory reactions of inappropriately activated PMN is well recognized [15, 16]. While activation due to infection results normally in the emigration of PMN into the infected sites, where they generate and release their cytotoxic mediators, the activation in the microvasculature can cause damage to the vascular bed, including enhanced permeability, endothelial cell damage, and vasculitis. Moreover, due to de novo synthesis of cytokines and surface receptors, the functional repertoire of PMN expands during the inflammation (reviewed in [15, 16]). While in Wegener's granulomatosis a participation of PMN in vasculitis and also in glomerulonephritis [17] is well established, the activating entities are not yet identified.

ANCA appeared to be good candidates, because of their association with active disease and because numerous in vitro studies described the ability of ANCA, and specifically of antibodies to PR3, to activate PMN and monocytes. Release of proinflammatory mediators,

including cytokines, oxygen radicals, prostanoids, and nitric oxide, was described, as was priming for higher responsiveness toward a secondary stimulus, and upregulation of activation-associated surface receptors [18–24].

Because binding of ANCA to PMN is central for their proposed role in Wegener's granulomatosis, the present study was designed to analyze the interaction of ANCA with PR3-expressing PMN. Binding of other autoantibodies to PMN, for example, to myeloperoxidase, elastase, and lactoferrin was not addressed in this study, nor were patients with vasculitis other than Wegener's granulomatosis.

METHODS

Patients

After approval by the Ethikkommission (Ethic committee) of the University Heidelberg Hospital and after having obtained informed consent, blood was taken from patients with Wegener's granulomatosis. Wegener's granulomatosis was defined as proposed by the Chapel Hill conference [25] and diagnosed according to the ACR criteria [26]. Disease activity was determined by the "Birmingham Vasculitis Activity Score" (BVAS) [27]; ANCA titers and PR3 titers were measured as reported elsewhere [28, 29]. Ten patients with active disease (BVAS >3) were included. From 64 patients with Wegener's granulomatosis, plasma samples (stored at −20°C) were used. All patients included in this study were negative for other autoantibodies (e.g., to nuclear antigens, myeloperoxidase, elastase or lactoferrin, or for rheumatoid factor). For comparison, whole blood, PMN, or plasma of healthy donors were used.

Cytofluorometry

To reduce artifacts due to storage or handling, surface-associated IgG and expression of PR3 were measured in whole blood, gathered in heparinized tubes (Saarstedt, Nümbrecht, Germany) within 1 hour after blood withdrawal. A polyclonal antibody to PR3, raised in rabbit (Elastin Products, Ownesville, MO, USA) (5 μL per 100 μL blood) was used and antirabbit IgG (from goat), fluorescein isocyanate (FITC)-conjugated (Jackson ImmunoResearch, purchased from Dianova, Hamburg, Germany), as was a monoclonal antibody to PR3 (PeliCluster, purchased from Höltzel Diagnostika, Köln, Germany) and a antimouse IgG-FITC (from goat) (Jackson ImmunoResearch). Binding of ANCA (or IgG) to PMN was measured by a FITC-labeled antibody to human IgG (from goat), recognizing all IgG subclasses (Jackson ImmunoResearch) and was measured as mean fluorescence intensity (MFI). For comparison, antimouse IgG FITC or antirabbit IgG FITC (also produced in goat) (Jackson ImmunoResearch) were used.

PMN were identified by expression of CD66b, monocytes using CD14 (Jackson ImmunoSearch); a gate was set around the CD66b- or CD14-positive cells, respectively, and 10,000 events were counted. Facscalibur® was used with Cellquest® as software (Becton and Dickinson, Heidelberg, Germany).

Experimental conditions to detect surface-associated ANCA. Whole blood (300 μL) was incubated either on ice or at room temperature with 1 to 10 μL of the antihuman IgG FITC for 20 minutes in the presence of 0.1% sodium azide to prevent internalization. Then the erythrocytes were lyzed using lyzing solution (Becton and Dickinson). The cells were resuspended in phosphate-buffered saline (PBS) (0.01 mol/L pH 7.4, containing paraformaldehyde 1%) and were subjected to cytofluorometry. For comparison antibodies to either mouse or rabbit IgG were used, as were untreated cells to control for the autofluorescence.

Of the isolated cells 1×10^5 to 1×10^6 were suspended in 300 μL "FACS" buffer [PBS 0.01 mol/L, pH 7.4, containing 1% bovine serum albumin (BSA) and 0.1% sodium azide]. Plasma as source of ANCA was used undiluted (100 μL), as was plasma of healthy donors. Of the IgG preparations (see below) 2 to 8 μg in a volume of 100 μL PBS was used, if not indicated differently. The cells were incubated with ANCA or IgG, respectively, for 20 minutes at 4°C or 22°C, then washed twice in FACS buffer, before adding the antihuman IgG FITC for another 20 minutes. To account for interindividual differences, each plasma or IgG sample was tested with PMN from three different donors. Since the results did not vary considerably, only one of the three results is shown in the figures or tables.

PMN isolation and induction of PR3 expression

PMN were isolated from heparinized blood by Polymorphprep™ (Nycomed, Oslo, Norway) followed by hypotonic lysis. This yielded 85% to 95% pure PMN. To induce PR3 expression, PMN were suspended in Hank's buffered salt solution (HBSS), pH 7.2, consisting in 10 mL 10 × Hank's, 275 μL 1 mol/L Tris HCl, pH 7.25, 170 μL 1.1 mol/L $CaCl_2$, 200 μL 0.4 mol/L $MgSO_4$, and 0.2 g glucose in 100 mL water) and incubated with phorbol-myristate-acetate (PMA) (Sigma, Deisenhofen, Germany), 1 μg/mL for 20 minutes at 37°C. PR3 expression was measured by cytofluorometry as described above. The polyclonal antibody (1 to 5 μL) and the monoclonal antibody to PR3 (1 to 2 μL) gave essentially similar MFI when tested on activated PMN of the same donor. Following preincubation with the polyclonal antibody for 15 minutes on ice, the binding of the monoclonal antibody was inhibited and vice versa.

Preparation of IgG from whole plasma

Protein A sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) was used and IgG was prepared according

Table 1. Expression of proteinase 3 (PR3) and binding of autoantibodies to neutrophil cytoplasmic antigens (ANCA) to polymorphonuclear neutrophils (PMN) of patients with Wegener's granulomatosis and active disease, or to PMN of healthy donors

Patient number	ANCA titer	Antibodies to PR3 in plasma	PR3 expression on PMN (detected by a polyclonal antiserum generated in rabbit)		ANCA on PMN (detected by antihuman IgG FITC)	
			% positive[a] PMN	MFI[a]	% positive PMN	MFI[a]
1	320	>100%	96.7	34	2.6	88
2	640	>100%	43.4	43	3.7	78
3	320	>100%	51.6	49	8.7	94
4	160	53.6%	87.5	54	12.7	56
5	80	87.3%	34.6	21	4.5	80
6	80	24.7%	78.7	31	14.7	69
7	160	>100%	100	49	4.0	56
Mean ± SD			70.6 ± 26.7	40.4 ± 11.9	7.3 ± 4.8[b]	74.4 ± 14.8[b]
Healthy donors ($N = 10$)		No data	22.1 ± 21.9	39.1 ± 13.0	14.9 ± 5.2	88.8 ± 12

MFI is mean fluorescence intensity; FITC is fluorescence isothiocyanate.

[a] % positive PMN or MFI were determined by cytofluorometry from experiments similar to those shown in Fig.1 using the Cellquest®program.

[b] The mean values do not differ significantly between the patients and the donor group [tested by two-sided *t* test or analysis of variance (ANOVA)].

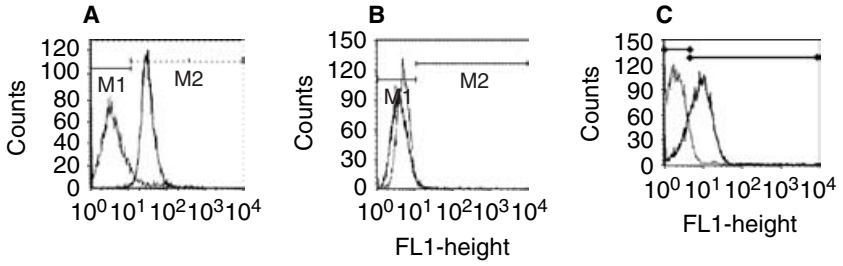

Fig. 1. Determination of proteinase 3 (PR3) expression on and binding of autoantibodies to neutrophil cytoplasmic antigens (ANCA) to polymorphonuclear neutrophils (PMN) derived from a patient with Wegener's granulomatosis. (*A*) PR3 was detected on PMN in whole blood [patient 1, with active disease; Birmingham Vasculitis Activity Score (BVAS 9)] by use of a polyclonal rabbit antibody to PR3 and fluorescein isothiocyanate (FITC)-labeled antirabbit IgG. The thin line shows the control (rabbit IgG plus FITC-labeled antirabbit IgG); the thick line anti-PR3. (*B*) From the same blood sample, an aliquot was incubated with antihuman IgG, FITC-labeled (thick line); for comparison unlabeled cells are shown (thin line). (*C*) Binding of the antihuman IgG (thick line) to erythrocytes in whole blood of a patient with "Coombs-positive" autohemolytic anemia is shown (the autoantibody was identified as anti-e Rhesus antibody); the patient had severe anemia as indicated by total hemoglobin 6.4 g/dL; lactate dehydrogenase (LDH) 700 U/L; haptoglobin <8 U/L. For comparison, antimouse IgG (produced in goat similarly to the antihuman IgG) was used (thin line).

to the protocol given by the supplier. Of each patient or of the healthy donors, respectively, 1 mL plasma was used and incubated over night at 4°C with constant shaking. Following repeated washing, IgG was eluted with 500 µL 0.1 mol/L citric acid buffer, pH 3.0, dialyzed against distilled water and finally against PBS. Protein concentration was measured photometrically. The IgG fraction of patients is termed "IgG-PR3-ANCA" in the following. In concentrations of 0.1 to 1 µg/mL, positive immunofluorescence with ethanol-fixed PMN was seen.

Statistics

Differences between mean values were calculated using *t* test for unpaired samples.

RESULTS

Expression of PR3 on PMN of patients with active Wegener's granulomatosis, but no evidence for binding of ANCA

PMN of seven patients with active Wegener's granulomatosis, ANCA titers ≥1:640 and autoantibodies to PR3, were analyzed by cytofluorometry (Table 1). To avoid artifacts due to the purification procedures, analysis was done in heparinzed whole blood. With a polyclonal antibody to PR3, raised in rabbit, PR3 was found on PMN of all the patients, and to a lesser extent on PMN of the healthy donors (data derived from one patient are shown in Fig. 1) (data of all patients and of the donors are summarized in Table 1). PR3 expression was also seen on the monocytes (data not shown).

Table 2. Direct comparison of binding of antibodies to IgG a 4°C or 22°C

Patient number	ANCA titer	Binding of antihuman IgG		Binding of antimouse-IgG	
		4°C	22°C	4°C	22°C
8	320	31.3[a]	34.5	20.9	22.3
9	320	33.9	24.2	25.7	15.4
10	80	19.0	45.9	17.5	18.7
Donors ($N = 3$)	No ANCA	28.4 ± 14.4	31.3 ± 16.0	24.5 ± 12.9	21.3 ± 14.8

ANCA is autoantibodies to neutrophil cytoplasmic antigens.
[a]Measured as mean fluorescence.

Because of the simultaneous presence of PR3 on PMN and autoantibodies to PR3 in the plasma, it was tested next whether IgG was bound to the patients' PMN. A FITC-labeled antibody to human IgG, reacting with all IgG subclasses was used and binding was compared to blood samples without any antibody added (to account for the autofluorescence), or to samples incubated with FITC-labeled antbodies to either mouse IgG or rabbit IgG in similar protein concentrations (Fig. 1). The mean fluorescence obtained with antihuman IgG (MFI 74.4 ± 14.8) did not differ from that obtained with antimouse IgG (66.9 ± 15.9) or antirabbit IgG (88.4 ± 25.0). Moreover, there was no difference in binding of antihuman IgG FITC to PMN of patients with active disease compared to PMN of healthy donors (Table 1). Of three additional patients (patients 8 to 10) two blood samples were taken. One was kept and processed on ice (4°C), the other at room temperature (22°C). There was no difference in antihuman IgG binding between the samples, indicating that temperature had no effect on anti-IgG binding (data summarized in Table 2).

That the antibody to human IgG was able to recognize surface-bound IgG was tested by using blood of a patients with "Coombs-positive" autoimmune hemolytic anemia, where binding of the antihuman IgG occurred to erythrocytes, but not of antimouse IgG (an example is shown in Fig. 1C).

Thus, from these data, there is no evidence for binding of PR3-ANCA to PR3 expressing PMN derived from the peripheral blood of patients with Wegener's granulomatosis.

Binding of PR3-ANCA to PMN of healthy donors

In another set of experiments, binding of PR3-ANCA to PMN of healthy donors was tested. The PMN were isolated from the peripheral blood and incubated with PMA to induce PR3 expression. Depending on the individual donor, PR3 expression was achieved on 70% to 100% of the PMN (mean 85 ± 10.2%, $N = 5$) as determined by use of the rabbit antibody to PR3 or the monoclonal antibody (an example is shown in Fig. 2A). The PMN were incubated with plasma from patients with Wegener's granulomatosis and for comparison with plasma of healthy donors. Binding of PR3-ANCA was determined by a FITC-labeled antibody to human IgG and measured as MFI. The MFI obtained with the individual plasma samples varied widely. In Fig. 2B, data for 42 patients with ANCA titers ranging from 1:20 to 1:1280 are shown in relation to the mean fluorescence. There was no correlation between the ANCA titer and the MFI. Moreover, when compared as groups, the MFI did not differ between plasma of patients with positive, high ANCA titer (≥1:160; anti-PR3 >100%; $N = 15$), or plasma of patients with Wegener's granulomatosis in remission and no ANCA (≤1: 20; no detectable anti-PR3; $N = 24$), or plasma of healthy donors ($N = 20$) (Fig. 2C).

That MFI occurred independently of the ANCA titer was also seen in follow-up studies. When plasma with high ANCA titer (obtained when the disease was active) was compared with plasma of the same patient taken after remission and when the ANCA titer had declined, nearly identical mean fluorescence was seen (data of one patient are shown Fig. 2D) (data of five patients are summarized in Table 3). Again, carrying out the binding assay on ice did not yield ANCA-dependent binding (an example is shown in Fig. 2D).

The data so far indicated that incubation of PMN with plasma caused a minor increase in the MFI, which was not dependent on the ANCA or more specifically, the anti-PR3 titer. In line with this finding, a monoclonal antibody to PR3 did not affect binding of ANCA-positive plasma to PMN, while, as expected, binding to PMN of the polyclonal rabbit anti-PR3 was inhibited by 40% to 50% (measured as reduction of MFI).

With essentially similar experiments, binding of ANCA to isolated monocytes of healthy donors was tested. Again, there was no evidence for a PR3-dependent binding (data not shown).

Binding of purified IgG-PR3-ANCA to PR3-expressing PMN of healthy donors

From the plasma of 25 patients with high ANCA titer (>1:640 and PR3 >100%), and from six donors, IgG was prepared, and binding of the IgG fraction, in the following termed IgG-PR3-ANCA, to PMA-treated PMN was tested. For 14 of the 25 patient-derived IgG preparations binding to PMN exceeding that of IgG obtained from healthy donors was seen, when 2 to 8 μg protein were used (Fig. 3).

Testing the specificity of binding did not yield entirely conclusive results. Preincubation of PMN with the monoclonal antibody to PR3 reduced binding of IgG-PR3-ANCA (34 ± 17%), as did mouse IgG (18 ± 12%) (mean of five experiments using IgG-PR3-ANCA derived from different patients). With all IgG-PR3-ANCA, however, inhibition by anti-PR3 was more pronounced, suggesting that at least a part of the IgG binding was PR3-specific.

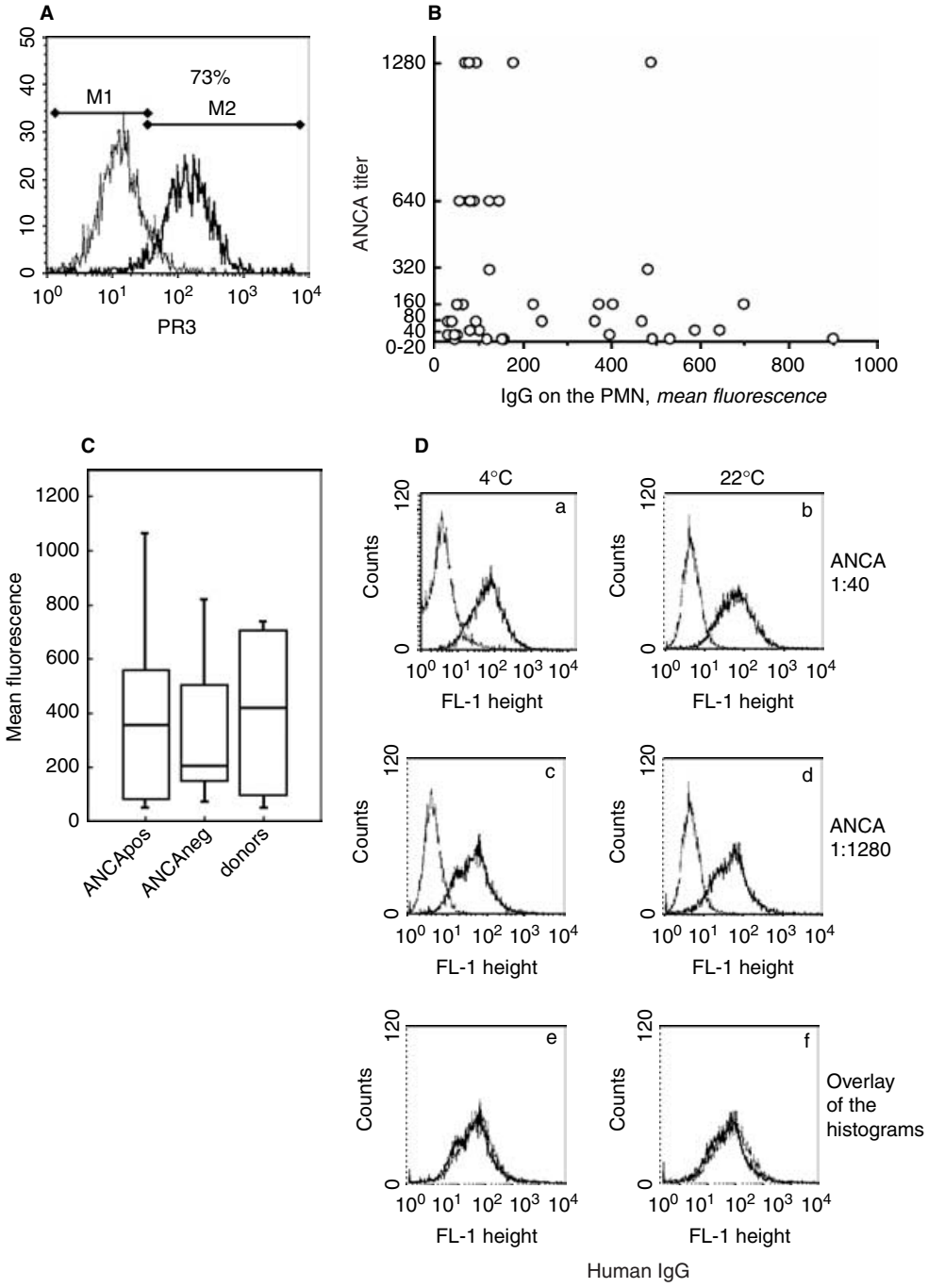

Fig. 2. Binding of autoantibodies to neutrophil cytoplasmic antigens (ANCA) to proteinase 3 (PR3)-expressing polymorphonuclear neutrophils (PMN) of healthy donors. (*A*) Incubation PMN of a healthy donor with PMA causes an up-regulation of PR3 on the surface (detected by antirabbit PR3, thick line, the thin line shows binding of rabbit IgG). In this experiment 73% of the donor PMN acquired PR3. (*B*) Binding of ANCA-containing plasma to PMN pretreated with PMA. The ANCA titer of the patient's plasma, ranging from 1:20 to 1:1280 ($N = 42$) is plotted (y axis) against the mean fluorescence (x axis) obtained after incubating PMN with the plasma. There is no correlation between the two values. (*C*) Binding of ANCA-containing plasma or control plasma to PMN pretreated with PMA. Binding data from 15 patients with ANCA titer >160; PR3 >100% (ANCA-positive) are summarized, as are data from 24 patients with ANCA <20 and anti-PR3 0 (ANCA-negative) and of 20 healthy donors. The boxes contain 50% of the values. There was no statistically significant difference between the three groups. (*D*) Plasma with ANCA (titer 1:1280; anti-PR3 >100%) was incubated with PMN at 4°C or 22°C (c and d), then secondary antibody [antihuman IgG-fluorescenin isothiocyanate (FITC)] was added (thick line); the thin line shows binding of the secondary antibody alone. For comparison, plasma of the same patient, taken during remission (ANCA titer 1:40; anti-PR3 11.3) was tested (a and b). The lower panel shows (e) the overlay of the histograms (a and c) and (f) of (b and d), respectively (for clarity, the isotype controls are not shown on the overlay).

Table 3. Binding to proteinase 3 (PR3)-expressing polymorphonuclear neutrophils (PMN) of patient-derived plasma compared with binding of plasma taken during acute disease, when the autoantibodies to neutrophil cytoplasmic antigens (ANCA) titer and anti-PR3 titers were high, with plasma taken of the same patient in remission

Patient number	ANCA titer	Anti-PR3	ANCA on PMN *mean fluorescence intensity*
330			
Acute disease	160	>100%	401
Remission	20	28%	403
434			
Acute disease	1240	>100%	489
Remission	40	11.3%	587
468			
Acute remission	80	76%	467
Remission	0	0%	527
373			
Acute remission	80	>100%	361
Remission	0	0%	496
326			
Acute remission	160	34%	49.9
Remission	0	0%	81.5

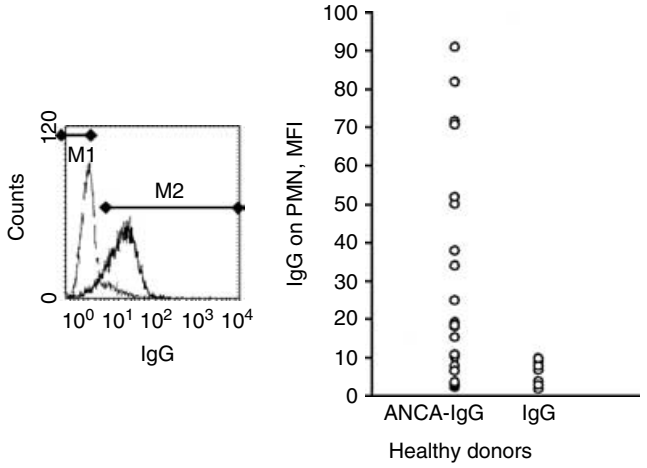

Fig. 3. Binding of IgG prepared from autoantibodies to neutrophil cytoplasmic antigens (ANCA)-containing plasma to polymorphonuclear neutrophils (PMN). Of 25 patients with ANCA titer of 1:640 or higher and proteinase 3 (PR3) titer >100% IgG was prepared. In concentrations of 4 μg/100 μL, IgG was added to phorbol-myristate-acetate (PMA)-activated PMN (3×10^5/300 μL). (*A*) Data of patient 1. The thick line represents binding of IgG-ANCA, the thin line donor IgG. (*B*) Binding of IgG-ANCA [measured as mean fluorescence intensity (MFI)] derived from 25 patients to donor PMN is shown, as is binding of IgG prepared from plasma of healthy donors ($N = 6$).

The observation that binding of isolated IgG-PR3-ANCA was seen while plasma failed to bind, despite high reactivity with ethanol-fixated PMN, suggested that the autoantibodies were of low affinity. Since direct determination of the affinity of polyclonal antibodies within an IgG preparation is not possible, various experiments were performed to indirectly get information on binding characteristics of the ANCA.

In a first set of experiments, the effect of heat-inactivated normal plasma on binding of IgG-PR3-ANCA to PMN was tested. With plasma concentrations

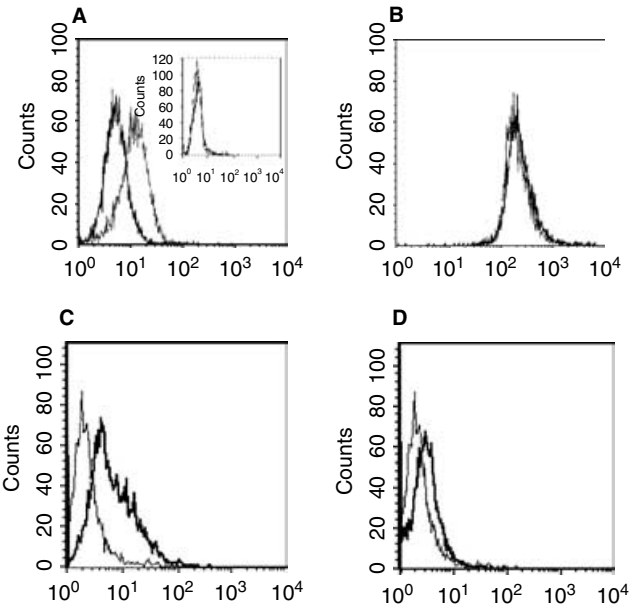

Fig. 4. Inhibition of IgG-autoantibodies to neutrophil cytoplasmic antigens (ANCA) binding to polymorphonuclear neutrophils (PMN). (*A*) IgG-ANCA was added to phorbol-myristate-acetate (PMA)-activated PMN (2 μg IgG protein in a total volume of 200 μL) in the absence (thin line) or presence of plasma (1% volume) (thick line) (the IgG controls are shown as inserts). (*B*) A similar experiment with anti-proteinase 3 (PR3) from rabbit. Here the two peaks coincide. (*C*) Binding of 2 μg IgG-ANCA in a total volume 200 μL (thick line) or 1000 μL (thin line) is shown. (*D*) Binding of IgG-ANCA in a total reaction volume of 1000 μL (thin line) is in the range of the IgG control (2 μg IgG isolated from a healthy donor; thick line).

as low as 1%, the binding of IgG-PR3-ANCA was reduced by 20% to 50% (mean 33.7 ± 12, $N = 12$) (an example is shown in Fig. 4). In the presence of higher plasma concentrations (5% to 10%) binding was totally inhibited. Also adding purified IgG or albumin (1 to 5 μg) inhibited binding of IgG-PR3-ANCA as did increasing the reaction volume. A doubling of the volume already reduced binding by 50%, increasing the volume fivefold, prevented binding entirely. Of note is that binding of the polyclonal rabbit IgG was not inhibited by plasma, or by increasing the reaction volume. Both sets of experiments indicate that the IgG-PR3-ANCA binding is of low affinity.

DISCUSSION

Since the detection of ANCA, the participation of those antibodies in the development and the progression of the disease has been investigated. Despite the facts that ANCA are not unique for Wegener's granulomatosis, that ANCA are not necessarily present in the initial phase of the disease or in localized disease, and that there are patients with Wegener's granulomatosis who are constantly negative for ANCA [30–32], there is an increasingly strong belief that Wegener's granulomatosis

is an autoimmune disease and that one of the underlying molecular mechanisms is the activation in the microvasculature of living PMN by ANCA [2, 3, 7–9].

The data presented here argue against this hypothesis, because binding of ANCA to its presumed target, the PR3-expressing PMN, could not be demonstrated. On peripheral blood PMN from patients with active Wegener's granulomatosis and autoantibodies to PR3, no surface-bound IgG could be detected, despite the fact that PR3 was expressed and bound the heterologous (rabbit) antibody. The most straightforward explanation is that ANCA, in contrast to the heterologous antibody, cannot bind to surface-associated PR3. The failure of ANCA to bind to intact PMN could be confirmed by in vitro experiments. When PMN of healthy donors, stimulated to express PR3, were incubated with ANCA-containing plasma of patients with Wegener's granulomatosis, only a minor increase in MFI was seen when FITC-labeled anti-IgG was added. This increase, however, was not dependent on the ANCA/anti-PR3 titer, nor could it be inhibited by a monoclonal antibody to PR3, the latter in contrast to binding of the polyclonal, rabbit-derived antibodies to PR3. The most likely interpretation is that the increase in fluoresecence was due to binding of plasma IgG in an antigen-unspecific manner, such as binding via its Fc portion to the Fc receptors on PMN.

In contrast to plasma, IgG fractions prepared from the ANCA-containing sera bound to PMN. For the majority of patients the binding of IgG-ANCA exceeded that of normal IgG, and could, at least in part, be inhibited by antibodies to PR3. Binding, however, was inhibited by plasma, by normal IgG or by albumin, and by increasing the reaction volume. These findings are compatible with low-affinity binding of PR3-ANCA.

Taken together, our data show that autoantibodies to PR3 fail to bind to viable PMN expressing PR3 under in vivo analogue conditions (i.e., in plasma), and that binding of the IgG-ANCA, if it occurred at all, was of low affinity.

Why ANCA fail to bind to intact PMN as measured ex vivo or when in whole blood, while binding to ethanol-fixed PMN or to soluble PR3 [e.g., as it used for the enzyme-linked immunosorbent assay (ELISA)] occurs is under investigation. Various, not mutually exclusive, explanations seem possible, including degradation of IgG by the surface-associated PR3, internalization of the immune complex, or blocking of the binding by plasma factors. We think that these explanations are unlikely because keeping the samples on ice, which should prevent degradation, did not allow surface detection of IgG. Moreover, degradation should be most efficient in the absence of plasma because of the absence of plasma-derived protease inhibitors; in our experiments, however, binding occurred with IgG-preparations, but not when plasma was present. Internalization is also not a convincing explanation; in vivo and in the experiments with whole blood ANCA, the PR3-expressing PMN coexist with the plasma autoantibody. Thus, a continuous binding of the autoantibody to PMN would be expected. By the same argument, also clearance of autoantibody-coated PMN is improbable, because neutropenia is not typically seen during active disease. Blocking or shielding of the PR3 binding sites could be possible, provided the blocking/shielding was selective for the autoantibody, and did not inhibit the heterologous antibody. While we cannot rule out this possibility, our data using purified IgG-PR3-ANCA show that binding can be inhibited not only by plasma but also by other sources of protein and by increasing the reaction volume as well.

We propose the explanation that epitopes recognized by the autoantibodies are not displayed by the surface-associated PR3. Experiments with denatured PR3 as antigen suggested a limited number of conformational epitopes [33], while more recent studies, using peptides, identified a restricted number of linear epitopes [34, 35]. It is possible that these epitopes are not accessible for the autoantibody when PR3 is folded, or posttranslationally modified or, as in the case of PR3 in vivo, is associated with the cell surface. Already minor modifications of the three-dimensional structure might be sufficient to reduce the number of possible interactions between PR3 and the autoantibody. When the epitope does not fit properly into the antibody's binding groove, low-affinity binding ensues, which is readily perturbed by other proteins (e.g., plasma proteins) or when the reaction volume is large. This, in turn, could explain why only purified IgG-PR3-ANCA can bind, and not the IgG in plasma or in whole blood. Heterologous antibodies (generated in rabbit or in mice) with high affinity, in contrast, can bind even in the presence of whole plasma or in larger reaction volumes. That autoantibodies are of low affinity, compared to antibodies obtained by immunization with a heterologous protein, is in keeping with tolerance to self-antigens and with the different route of antigen presentation.

At first glance, our data appear to contradict the wisdom accumulated over the years; at a second glance, however, they concur with published data, though not necessarily with the conclusions. First, our data are consistent with the histomorphologic characterization of the inflammatory lesions as "pauci immune," a term coined to describe the absence of immunoglobulins, but not of cellular infiltrates, the latter being abundant. In patients with glomerulonephritis, for example, in biopsies massive infiltration of PMN was seen and also PR3, but no IgG, despite high autoantibody titers [17]. To detect one without the other is reminiscent of the data with peripheral blood PMN of patients with active disease, and until proven differently, the most reasonable explanation is that ANCA do not bind to PMN in vivo.

Another argument against binding of ANCA in vivo is the observation that neutropenia is not typically seen in Wegener's granulomatosis, nor is a decrease of complement factors, which one would expect as result of complement activation by surface-bound IgG. As known from true autoimmune diseases or from acquired neutropenia, antibody-coated PMN are cleared rapidly from the circulation [36]. One example for a presumed ANCA-mediated neutropenia was described in a patient with Graves' disease, who, following treatment with propylthiouracil, developed ANCAs. In this patient specific binding to intact PMN of autoantibodies to PR3 and to myeloperoxidase was described, which induced cytotoxicity by an antibody- and complement-mediated process [37]. Because of the different mode of autoantibody induction, is it presumed that in this patient ANCA with high affinity are generated, in contrast to the low-affinity ANCA in patients with Wegener's granulomatosis.

One of the major arguments for an involvement of ANCA in the pathogenesis of vasculitis are the results reported lately about an antibody to myeloperoxidase causing glomerulonephritis in a mouse strain [38]. It should be emphasized that this study is concerned only with PR3-ANCA and its link to Wegener's granulomatosis and does not address the issue of myeloperoxidase-ANCA or microscopic polyangiitis.

Another argument for a role of ANCA in Wegener's granulomatosis is its good correlation to disease activity. Considering, however, that antibody de novo synthesis requires expansion and differentiation of the respective B-cell clones, it is quite feasible that autoantibodies occur as a consequence of an inflammatory response, rather than to create it.

Yet another argument for a role of ANCA in the pathogenesis of Wegener's granulomatosis is derived from in vitro experiments describing activation of PMN by ANCA. To the best of our knowledge and after extensive review of the literature, the in vitro experiments were carried out with IgG fractions of ANCA, with affinity-purified antibodies, or with heterologous antibodies. That IgG-ANCA or heterologous antibodies bind to PMN is in agreement with our data, although in our hands binding was of low affinity, not entirely specific for PR3, and did not occur with all IgG-ANCA preparations.

Nevertheless, that binding of IgG-ANCA to PMN activates the cells is not debated here, nor do we have any doubts that activated PMN are relevant effector cells for the development of vasculitis or of kidney damage. Moreover, we do not question the value of ANCA as a diagnostic tool. Challenged here is the hypothesis that PR3-ANCA are efficient activators of PMN in vivo, which consequently sheds some doubt on the role of antibodies to PR3 in the pathogenesis of Wegener's granulomatosis, at least as far as leukocyte activation in whole blood is concerned.

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